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DOI: https://doi.org/10.1016/j.soilbio.2016.11.001

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Soil Biology and Biochemistry

Vol. 104, January 2017, Pages 218–225

http://dx.doi.org/10.1016/j.soilbio.2016.11.001

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Abstract

Soil ecosystems actively regulate climate by controlling methane and nitrous oxide fluxes into the atmosphere. Soils have been, however, drastically altered by agricultural practices, such as nitrogen amendment which increases nitrous oxide emission while it reduces methane uptakes in well-aerated soils by affecting methane-oxidizing bacteria. New nitrification inhibitors, such as 3,4-dimethylpyrazole phosphate (DMPP), are often applied in combination with nitrogen-based fertilizer to increase plant productivity by increasing available ammonium and inhibiting denitrification processes reducing in turn nitrous oxide emissions. However, the increase in ammonium due to nitrification inhibition might also affect methane oxidizing bacteria. We therefore investigated the effects of nitrogen-based fertilizer and DMPP on methane and nitrous oxide fluxes in an extensively managed grassland. We also determined the spatial distribution of active methane oxidizing bacteria by radiolabeling. Short-term reduction in methane uptake and methanotrophic activity occurred after application of 600 kg N ha^{-1} while DMPP did not alter methane uptake but reduced nitrous oxide emission. The combination of both radiolabeling and field measurement revealed that methane uptake collapsed in the field when methanotrophic activity was inhibited not only in the surface but also in deeper soil. Finally, both methane uptake and methanotrophic activity recovered with time.

1. Introduction

Atmospheric methane (CH_4) and nitrous oxide (N_2O) are the most important anthropogenic greenhouse gases contributing to global warming after carbon dioxide (CO_2). Since the beginning of the industrial era, atmospheric concentrations of CH_4 and N_2O have increased by 150% and 20%, respectively, mainly due to anthropogenic emissions, and currently account for more than ¼ of anthropogenic radiative forcing (Solomon et al., 2007).

Soils can be both sources and sinks of CH_4 (Conrad, 1996). CH_4 is primarily produced when organic matter is degraded under anaerobic conditions. Recently, aerobic CH_4 production through non-microbial processes has also been observed (Keppler et al., 2006; Wang et al., 2013), but the quantitative importance of these fluxes in natural ecosystems remains unclear. CH_4 is removed from soils through microbial oxidation, and by diffusion to the atmosphere where it is photochemically degraded. The soil CH_4 sink is essentially driven by methanotrophic bacteria, which use CH_4 as carbon and energy source. Soil CH_4 concentrations differ by orders of magnitude depending on whether CH_4 originates from the atmosphere or from soil-internal sources. Accordingly, two different apparent CH_4 oxidation kinetics are observed, with a "high affinity" process driving uptake from the atmosphere (Hanson and Hanson, 1996). However, the nature of the methanotrophs responsible for "high affinity" CH_4 oxidation remains enigmatic, since these organisms have not successfully been isolated to date (Dunfield, 2007). Although atmospheric CH_4 removal rates are low per unit ground area, many different ecosystems covering large land areas contribute to this flux, so that global atmospheric CH_4 sequestration on land accounts for an estimated 30 ± 6 Tg CH_4 per year (Le Mer and Roger, 2001).

N_2O is emitted from terrestrial soils as an intermediate or by-product of microbial N transformations, in particular nitrification and denitrification. The application of synthetic fertilizers or manure to soils accelerates N cycling and associated N_2O emissions. Globally, fertilized agricultural soils contribute approximately 65% to anthropogenic N_2O emissions (Solomon et al., 2007). The application of N-fertilizers, in particular when they are ammonium-based (NH_4^+), often also affects soil CH_4 uptake. However, response patterns are equivocal, and so are the underlying mechanisms. Some studies have reported an immediate decline of the soil CH_4 sink, an effect that has often been attributed to competitive inhibition of the methane mono-oxygenase enzyme system by NH_3 (Bedard and Knowles, 1989), but the production of toxic by-

products (NH_2OH and NO_2^-) during nitrification (King and Schnell, 1994) or osmotic effects (Price et al., 2004) may also be important. However, in the field, delayed or positive effects of NH_4^+ application on soil CH_4 uptake have also been found (reviewed in Bodelier, 2011), hinting at more complex mechanisms.

Agricultural N fertilizers often are applied in combination with nitrification inhibitors such as dicyandiamide (DCD) or 3,4-dimethylpyrazole phosphate (DMPP). DMPP specifically inhibits ammonium mono-oxygenase. A reduction in the activity of this key enzyme of NH_4^+ oxidizers reduces NO_3^- production through lower rates of NH_4^+ oxidation and subsequent nitrification, increases the amount of NH_4^+ available to plants, and ultimately decreases N_2O emission from soils. Ammonium mono-oxygenase is structurally very similar to the methane mono-oxygenase enzyme of methanotrophs, and both enzymes share – although with different affinity – a range of substrates. One could thus expect detrimental effects of DMPP on methanotrophs as well, but the available studies indicate no such reduction in methanotrophic activity or even a positive effect on soil CH_4 uptake, possibly due to a reduction in toxic by-products of nitrification (Weiske et al., 2001; Zerulla et al., 2001).

Understanding enzyme and cell-level mechanisms such as the inhibition of methane mono-oxygenase is important, but the effects they cause at small spatial scales often do not propagate one to one to the ecosystem level. Accordingly, laboratory responses of CH_4 oxidation to experimental treatments often differ from those of soil—atmosphere fluxes observed under field conditions. A crucial factor linking physiology to ecosystem-level processes is soil structure and the spatial organization of the active methanotrophic communities within this structure. In well-aerated soils, biological CH_4 oxidation is mainly limited by CH_4 and O_2 diffusivity, which in turn depends on soil texture, aggregation, porosity and moisture (del Grosso et al., 2000; Hartmann et al., 2011; Hiltbrunner et al., 2012; Young and Ritz, 2000). By using a novel radio-labeling technique that allowed the in situ spatial mapping of active methanotrophs, Stiehl-Braun et al. (2011a) found inhibition of soil CH_4 oxidation in top soil layers; this loss of function, however, was not evident at the ecosystem-level because deeper soil layers were able to compensate for the "failure" of the top layers, at least under drought conditions when CH_4 diffusivity was high and CH_4 could diffuse to deep soil layers at sufficient rates. Long-term mechanisms, however, are more complicated. Once ecosystems lose their capacity to oxidize atmospheric CH_4 for extended periods, for example after fertilization (Hütsch et al., 1994) or land use change (Prieme

et al., 1997), recovery may be very slow (Prieme et al., 1997; Smith et al., 2000), and to date the mechanisms involved are not well understood (Hiltbrunner et al., 2012).

We investigated effects of NH_4^+-based fertilizer, in factorial combination with DMPP application, on CH_4 and N_2O fluxes in an extensively-managed and well-aerated grassland soil in Switzerland. N fertilizer was applied at different rates to quantify dose-effect relationships. Our goal was to analyze the temporal dynamics of CH_4 and N_2O soil-atmosphere fluxes and their relation to mineral N transformations and the active domains where CH_4 oxidation occurred within soil structure. We thus combined system-level CH_4 and N_2O flux measurements with time-series of laboratory soil analysis, and the high-resolution spatial mapping of methanotrophic activity in intact soil cores, using a radiolabeling technique (Stiehl-Braun et al., 2011a; Stiehl-Braun et al., 2011b). Specifically, we were interested in (1) testing whether soil CH_4 uptake is affected by N fertilization, and whether this effect depends on DMPP-application; and (2) analyzing the relation between local inhibition of CH_4 oxidation and ecosystem-level CH_4 fluxes, in particular with respect to the stability of system-level functioning under disturbance.

2. Materials and Methods

2.1. Experimental design

In June 2011, we set up a field experiment in which we factorially applied N fertilizer and a nitrification inhibitor. The study was located on an extensively managed natural grassland that has not been fertilized for at least 20 years. The grassland was mown one to two times each year (Agroscope Reckenholz Research Station near Zürich, Switzerland). The experiment was laid out as randomized split-plot design replicated in four blocks. Each block consisted of three 2 × 1 m plots, to which 0, 200 or 600 kg N ha^{-1} were applied. Each plot was subdivided into two 1 × 1 m subplots, to which either a nitrification inhibitor was applied or which served as control. All subplots were separated by 20 cm buffer stripes to prevent fertilizer and nitrification inhibitor to spread into adjacent plots.

Nitrogen fertilizer was applied on 6 June 2011 as 1 L ammonium sulphate [$(NH_4)_2SO_4$] solution. For the subplots treated with the nitrification inhibitor, the fertilizer solution also contained 3,4-dimethylpyrazole phosphate (DMPP, K+S Nitrogen GmbH, Mannheim, Germany) in amounts equivalent to 2% of the total N applied. After application of the solution, 2 L of water were applied per subplot to wash the fertilizer into the soil.

Soils were Cambisols and pH did not vary with depth (7.2 ± 0.2). C and N contents decreased with depth (C: 4.4 ± 1.3, 4.4 ± 2.1, 3.0 ± 0.8 %; N: 0.4 ± 0.1, 0.3 ± 0.1, 0.2 ± 0.1 %, in 0–5, 5–10 and 15–20 cm depth, respectively).

2.2. CH_4 and N_2O fluxes

We measured soil-atmosphere fluxes of CH_4 and N_2O 1, 4, 16, 25, 32 and 380 days after fertilizer application. A static chamber (32 cm diameter × 31 cm height) had been installed in the center of each subplot. Several weeks prior to fertilizer application, the chamber was lowered into the soil after cutting the ground with a spade, resulting in a final chamber height of 11 cm above ground. For the flux measurements, the chamber was closed with a lid and headspace samples collected after 5, 20 and 35 minutes using a 20 mL syringe. These samples were injected into pre-evacuated vials (Labco Limited, Buckinhamshire, UK) and CH_4 and N_2O concentrations measured (Agilent 7890N gas chromatograph with flame ionization and electron capture

detectors, Agilent, Wilmington, Delaware). CH_4 and N_2O flux rates were calculated by linearly regressing gas concentrations against sampling time. The fit was generally very good with a low residual standard error; r^2 exceeded 0.95 except for very small fluxes for which r^2 approaches zero for mathematical reasons.

2.3. Soil incubation

We collected two soil cores per plot 1, 4 and 16 days after fertilizer application, using a 5 cm diameter corer. These cores were divided into 0–5, 5–10 and 10–15 cm depth layers and sieved (2 mm mesh size). Approximately 100 g fresh soil per subplot were incubated at 20°C in 0.9 L gas-tight jars equipped with a septum. Headspace samples were collected 0, 18 and 36 hours after jar closure and analyzed for CH_4 and N_2O as described above. At the end of the incubation, the soil moisture of all samples was determined gravimetrically (24 h, 105°C) to calculate CH_4 and N_2O flux rates per dry soil mass.

Parallel to the gas flux measurements, soil NH_4^+ and NO_3^- concentrations were determined by extracting 5 g fresh sieved soil with 20 mL 2 M KCl (120 rpm, 1 hour on a table shaker). The resulting slurries were sedimented for 5 minutes, supernatants filtered (Whatman White Ribbon paper, Sigma-Aldrich, Germany), and NH_4^+ and NO_3^- concentrations determined colorimetrically (San++ automated wet chemistry analyzer, Skalar Analytical B.V., Breda, Netherlands).

2.4. Spatial mapping of methanotrophic activity

We mapped the spatial distribution of methanotrophic activity using a micro-autoradiographic method (see Stiehl-Braun et al., 2011a, for details). In brief, intact soil cores were collected 1, 4, 16 and 92 days after fertilizer application by inserting a 62 mm inner diameter PVC tube (PN6 tube, Debrunner Acifer AG, Zürich, Switzerland) into pre-cut ground. Each tube was carefully excavated from the side, capped at both ends, and transferred to the laboratory where the top cap was removed and the core placed upright into a 3 L gas-tight jar fitted with a septum. A vial with 40 mL 1 M NaOH was also placed in the jar to trap CO_2 produced during the subsequent incubation. The jar was then closed and the soil incubated for 10 days, during which a total of 100 kBq $^{14}CH_4$ was added. CH_4 concentrations were regularly monitored, and $^{14}CH_4$ added in small portions so that the CH_4 concentration always stayed below 10 ppm. To keep conditions aerobic, we also added 20 mL O_2 to each jar every two days.

166 The ^{14}C-labeled soil cores were frozen (-20°C) and freeze-dried before they were impregnated
167 with an epoxy resin (Laromin C 260 resin, BASF, Ludwigshafen, Germany, mixed at a 2:3 v/v
168 ratio with Araldite DY 026SP hardener, Astorit AG, Eisiedeln, Switzerland). To ensure complete
169 infiltration of the resin, the soil cores were placed in a desiccator that was slowly evacuated to
170 500 Pa pressure. Then, the pressure was slowly brought back to atmospheric levels. The
171 impregnated cores were left hardening at room temperature for four days before they were
172 incubated overnight at 60°C for complete resin curing. Each core was then cut lengthwise using a
173 diamond saw. The vertical section was divided horizontally in three 1 × 5 × 5 cm slides
174 corresponding to 0–5, 5–10 and 10–15 cm depth layers. Each slide was mounted on a glass
175 carrier and levelled with a diamond cup mill (Discoplan TS, Struers GmbH, Birmensdorf,
176 Switzerland). Thereafter, autoradiographies were obtained by placing the slides on phosphor
177 imaging plates (BAS IIIS, Fuji Photo Film, Tokyo, Japan) and exposing them for six days. The
178 plates were then digitized at a resolution of 200 μm with a red excited blue fluorescence laser
179 scanner (BAS 1000, Fujix Ltd., Kyoto, Japan). Background exposure was subtracted from the
180 data and the three slides recomposed to a single section of the original soil core (custom Matlab
181 scripts using the Image Processing Toolbox, Mathworks, Natick, MA). Vertical labelling profiles
182 were obtained using ImageJ (Wayne Rasband, National Institutes of Health, U.S.A).

183 ## 2.5. Statistical analyses

184 Data were analyzed by fitting mixed-effects models by maximum likelihood (ASReml 3.0,
185 VSN International, Hemel Hempstead, UK). Fixed effects were block, followed by N fertilizer,
186 DMPP application, and the N fertilizer × DMPP interaction. Random effects were plot and
187 subplot, and for the analysis of sieved soil samples, additionally also plot × soil layer and subplot
188 × soil layer. For repeated measures, we included a 1^{st} order autoregressive temporal correlation
189 of residuals. The repeated measures model included the additional random effects plot × date,
190 subplot × date, and, for the analysis of incubated sieved soil, plot × date × layer. Soil CH_4 uptake
191 rates were analyzed untransformed, whereas N_2O emissions were analyzed as log(N_2O flux rate
192 + 1 nmol N_2O m^{-2} d^{-1}) to account for the near-exponential distribution of residuals and some
193 small negative values which most likely were a result of measurement error.

3. Results

3.1. Soil-atmosphere CH_4 and N_2O fluxes

Soil CH_4 uptake was reduced by N fertilization at all sampling dates except for the measurement one year after N application (Fig. 1a, $P < 0.01$ for N, $P < 0.001$ for N × date). The N fertilizer effect was mainly driven by the highest application level (600 kg N ha^{-1}), whereas application at lower rates (200 kg N ha^{-1}) only tended to reduce soil CH_4 uptake after 16 days. DMPP application did neither change soil CH_4 uptake nor did it interact with N fertilizer application.

N_2O emissions increased under N application except for the last measurement after one year (Fig. 1b, $P < 0.001$ for N and date × N). N_2O emissions were reduced under DMPP application 25 and 32 days after application ($P<0.01$ and $P<0.05$, respectively), and this effect was mainly driven by the 600 kg N ha^{-1} application, with no significant effects detected when only 200 kg N ha^{-1} were applied ($P<0.05$ for N×DMPP for both dates).

3.2. Soil CH_4 and N_2O fluxes from incubated sieved soils

CH_4 uptake of sieved soil decreased with nitrogen application (Fig. 2a). This effect was driven by reductions in the 200 kg N ha^{-1} application relative to the unfertilized control (-66%, average across all dates and DMPP treatments), and even larger effects when 600 kg N ha^{-1} were applied (-97%). Net CH_4 oxidation decreased with depth when no N fertilizer was applied; however, this pattern reversed under N application, which inhibited CH_4 oxidation mostly in the top layers ($P < 0.001$ for N × layer). Nitrogen application effects also were time-dependent, with the zone of reduced CH_4 oxidation under N application progressively extending downwards ($P < 0.05$ for N × date; Fig. 2a). DMPP application did not significantly affect net CH_4 uptake rates (+3%, n.s.).

N_2O production from sieved soil increased with N application rate ($P < 0.001$, Fig. 2b), and this increase was larger in top soil layers than deeper down the soil profile ($P < 0.01$ for N × layer). DMPP application did not affect N_2O production in these assays (-5%, n.s.).

Net CH_4 uptake of incubated sieved soils was negatively correlated with soil NH_4^+ concentrations, with a strong non-linear component. Combining all samples, the relationship could well be described with a negative hyperbola (Fig. 3), or as linear relationship of CH_4

uptake with log([NH_4^+]) (r^2 = 0.62). Soils generally were a net CH_4 sink when NH_4^+ concentrations were below 0.5 mg NH_4^+-N (g soil)$^{-1}$, and turned into a weak CH_4 source at higher concentrations. Soil NH_4^+ concentrations explained a large fraction of the variation in CH_4 uptake, with effects of N application levels no longer being significant when NH_4^+ concentrations were fitted first in our mixed-effects models. We also fitted soil moisture as covariate in the linear mixed-effects models, but it showed little variation (0.18 ± 0.03 g H_2O (g soil)$^{-1}$, mean ± s.d. of all samples) and was unrelated to CH_4 and N_2O fluxes.

3.3. Soil mineral N concentrations

Soil NH_4^+ concentrations were generally highest in the top soil layers ($P < 0.001$, Fig. 4) and increased with N fertilizer application rate ($P < 0.001$), mostly so in the top soil ($P < 0.001$ for N × layer). All these effects were time-dependent ($P < 0.001$ for date, N × date, layer × date and N × layer × date). Soil NO_3^- concentrations showed similar patterns as NH_4^+ (Fig. 5), but effects of N × layer × date were not statistically significant.

DMPP application did not reveal an effect on soil NH_4^+ concentrations in the repeated measures analysis. However, significant N × DMPP × layer interactions were found 4 ($P < 0.01$) and 16 ($P < 0.05$) days after treatment application. DMPP also significantly reduced soil NO_3^- concentrations ($P < 0.01$, repeated measures analysis), an effect which was evident on day 4 ($P=0.07$) and day 16 ($P < 0.01$, plus $P < 0.05$ for N × DMPP × layer) when dates were analyzed separately.

3.4. Spatial distribution of net CH_4 assimilation

The autoradiographies of soil sections showed a heterogeneous distribution of net $^{14}CH_4$ assimilation (Fig. 6). In control plots to which no nitrogen had been applied, CH_4 assimilation was evident in all soil layers except the top ~1 centimeter on day 1 and deeper soil layers when soils were wet after 92 day.

Nitrogen application resulted in a zone of inhibited CH_4 assimilation (dark red areas in Fig. 6) that progressively developed from top to bottom, reaching ~5 cm depth 4 days after fertilizer application at both N application rates. When 600 kg N ha^{-1} yr^{-1} were applied, this zone continued to develop until it covered nearly the entire soil profile assessed after 16 days. In

contrast, DMPP application had no effect on the spatial distribution of methanotrophic activity in the incubated soil cores.

Inhibition of $^{14}CH_4$ assimilation not only progressed vertically but also at a finer spatial scale within soil layers, presumably within soil aggregates. At depths that clearly were affected by N application, small areas showing methanotrophic activity remained at day 1 but these were reduced by day 4 and had largely disappeared by day 16. After 92 days, CH_4 assimilation had recovered and patterns of CH_4 assimilation were similar in plots having received N application and control plots. Overall this process resulted in an increasingly distinct separation of active and inactive spatial domains.

4. Discussion

Our study demonstrated effects of N fertilizer application on CH_4 oxidation at all levels investigated. Soil CH_4 uptake was reduced in the traditional static chamber measurements conducted in the field. CH_4 uptake also was reduced in sieved soil samples, and our autoradiographic analysis showed zones with virtually complete inhibition of CH_4 assimilation. However, the effects observed at these different scales were not directly correlated, providing insight into the mechanisms controlling system-level CH_4 dynamics.

The autoradiographic analyses clearly showed that N fertilizer application reduced the assimilation of CH_4 in the upper soil layers, a finding which was confirmed by laboratory incubations of fresh sieved soil sampled from these depths. The fact that responses were similar also indicates that low $^{14}CH_4$ assimilation was not due to diffusive limitations, at least not at spatial scales above single aggregates, since these limitations were ineffective after sieving. Interestingly, these local (top soil) reductions in CH_4 assimilation did not translate into a reduced ecosystem-level soil CH_4 sink except at the largest application of 600 kg N ha^{-1}. Our findings thus highlight the importance of the spatial integration of processes for safeguarding the stability of ecosystem-level functioning. Based on our results, we argue that local losses of methanotrophic activity can be compensated by increased CH_4 oxidation rates in other soil domains. This stabilizing mechanism appears to be effective even if the inhibited zones are quite large, covering the entire top five centimeters of the soil. These findings are in line with Stiehl-Braun et al. (2011a) who reported similar effects in managed grassland fertilized with 150–200 kg N ha^{-1} yr^{-1}. In their study, however, effects only occurred under drought, most likely because ammonium was effectively removed when sufficient water was available, either by plant assimilation or by nitrification (Hartmann et al., 2013; Hartmann and Niklaus, 2012), and inhibitory effects thus did not develop under normal water supply. In the present study, no severe drought occurred in the month after fertilizer application, although soil moisture was low as is typical for that time of the year. It therefore appears possible that inhibitory effects of fertilizer on CH_4 fluxes would also have been found at a lower N fertilization level in our study if NH_4^+ had accumulated under drought.

Soil CH_4 oxidation of isolated, sieved soil layers correlated strongly with NH_4^+ concentrations; virtually no CH_4 was consumed when NH_4^+ concentrations exceeded

289 approximately 0.5 mg NH_4^+-N (g soil)$^{-1}$. At lower NH_4^+ concentrations, a negative correlation
290 with CH_4 oxidation was found, but there also was a high degree of variability around the mean
291 relationship. To some degree, this scatter might have been due to random variation reflecting the
292 accuracy in particular NH_4^+ concentrations measurements. However, we consider it likely that
293 limiting factors other than NH_4^+ were also at play. Soil moisture often controls CH_4 oxidation
294 rates (Dörr et al., 1993), either through diffusive limitation at high water contents or through
295 water limitations at low water content (Kammann et al., 2001), but we were not able to identify
296 such a relation with the given data. Such a co-limitation would ideally be investigated by
297 experimentally manipulating soil moisture, either in the field or of sieved samples, but we did
298 not do this in our study. The correlation we found between soil CH_4 uptake and NH_4^+
299 concentration is compatible with a putative enzymatic inhibition mechanism (Bodelier and
300 Laanbroek, 2004), but we argue that – while apparently intuitive – other mechanisms may also
301 have been involved. For example, general osmotic effects of non-N salts can manifest similarly
302 (Price et al., 2004; Whalen, 2000).

303 N_2O emissions increased strongly with N application levels, in line with similar field trials
304 (e.g. Acton and Baggs, 2011), reflecting the general consensus implemented e.g. in the emission-
305 factor based IPCC standard methodology to determine N_2O emissions (Eggelston et al., 2006).
306 Increased N_2O emissions were evident both at the ecosystem level and in sieved soil samples.
307 The latter are difficult to relate to field conditions since redox potential changes when soils are
308 sieved and well-aerated; nevertheless, our data suggest that the top 10 cm of soil contributed
309 most to these emissions. The increases in N_2O emissions likely were caused by combined
310 increases in nitrification and subsequent denitrification.

311 In our study, application of the nitrification inhibitor DMPP did not affect any measured field
312 or laboratory CH_4 flux. This suggests that DMPP did not affect methanotrophic bacteria, neither
313 through direct effects on their enzyme system nor indirectly through the accumulation of NH_4^+
314 concentrations above critical levels. We did not detect DMPP-related NH_4^+ concentration
315 changes in our study, although NO_3^- concentrations and N_2O emission decreased shortly after
316 application of the nitrification inhibitor. However, soils are highly heterogeneous, with
317 nitrification and even more so denitrification taking place in particular soil domains, at particular
318 times when substrate availability and redox conditions are favorable. It may well be that DMPP
319 application curbed such localized and episodic excessively high NH_4^+ peaks. Such isolated

effects likely go unnoticed in bulk soil measurements, although they have the potential to substantially reduce the frequency of hot spots and hot moments that generally account for a large fraction of soil N_2O emissions.

Changes in net soil-atmosphere CH_4 fluxes reflect the combined effects on methanotrophs and methanogens. It is thus difficult to attribute effects to specific processes when both communities contribute to CH_4 dynamics. Only a few studies have addressed effects of nitrification inhibitors in such systems (Datta and Adhya, 2014; Luo et al., 2013; Mohanty et al., 2009; Pereira et al., 2010), and effects mediated by changes in methanotroph and methanogen communities have thus rarely been disentangled. An exception is the study by Datta and Adhya (2014) who found 50% increased CH_4 emissions from rice paddies when dicyandiamide (DCD), a commercial nitrification inhibitor, was applied. The authors related this increase in emissions to increased N availability due to the high N content of DCD. However, this effect was also related to lower counts of methanotrophs while methanogens remained unaffected, suggesting that increased CH_4 emissions might also have resulted from reduced oxidation of CH_4. A strong drop in methanotroph community size after the application of DCD was also found by Mohanty et al. (2009).

Our study addressed effects of a one-time application of fertilizer and a nitrification inhibitor. The available data suggests that microbial activities had reverted to control conditions after several months. Effects of repeated, long-term application may however be different. We think that single applications mainly caused a transitory inhibition of methanotrophic activity. If mortality occurred, then the remaining viable community at these particular locations was able to maintain CH_4 oxidation rates, and communities would be likely to regenerate in the longer term. However, sustained impacts of fertilizer application may lead to the complete eradication of methanotrophs from microsites, with recovery taking much longer and involving dispersal and meta-population dynamics. These processes are currently not well understood, and difficult to separate from effects of changes in micro-environmental conditions, including pH and redox potential (Hiltbrunner et al., 2012; Hütsch et al., 1994; Prieme et al., 1997; Stiehl-Braun et al., 2011b). Techniques such as the micro-autoradiography adopted here, or secondary ion mass spectroscopy (SIMS) may help to elucidate these spatial dynamics.

Acknowledgements

We are grateful to the Agroscope Reckenholz-Tännikon Research Station (ART) and Andreas Lüscher who allowed us to conduct our experiment on their fields. We thank René Husi, Andrea Schifferli, Anna Kolly for their help with laboratory analysis and field work. Frowin Pirovino (Geological Institute, ETH Zürich) is gratefully acknowledged for supporting this project by providing access to the tools necessary for soil core preparation. We also thank Sabine Ragot for useful comments on an earlier version of this manuscript.

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Figures

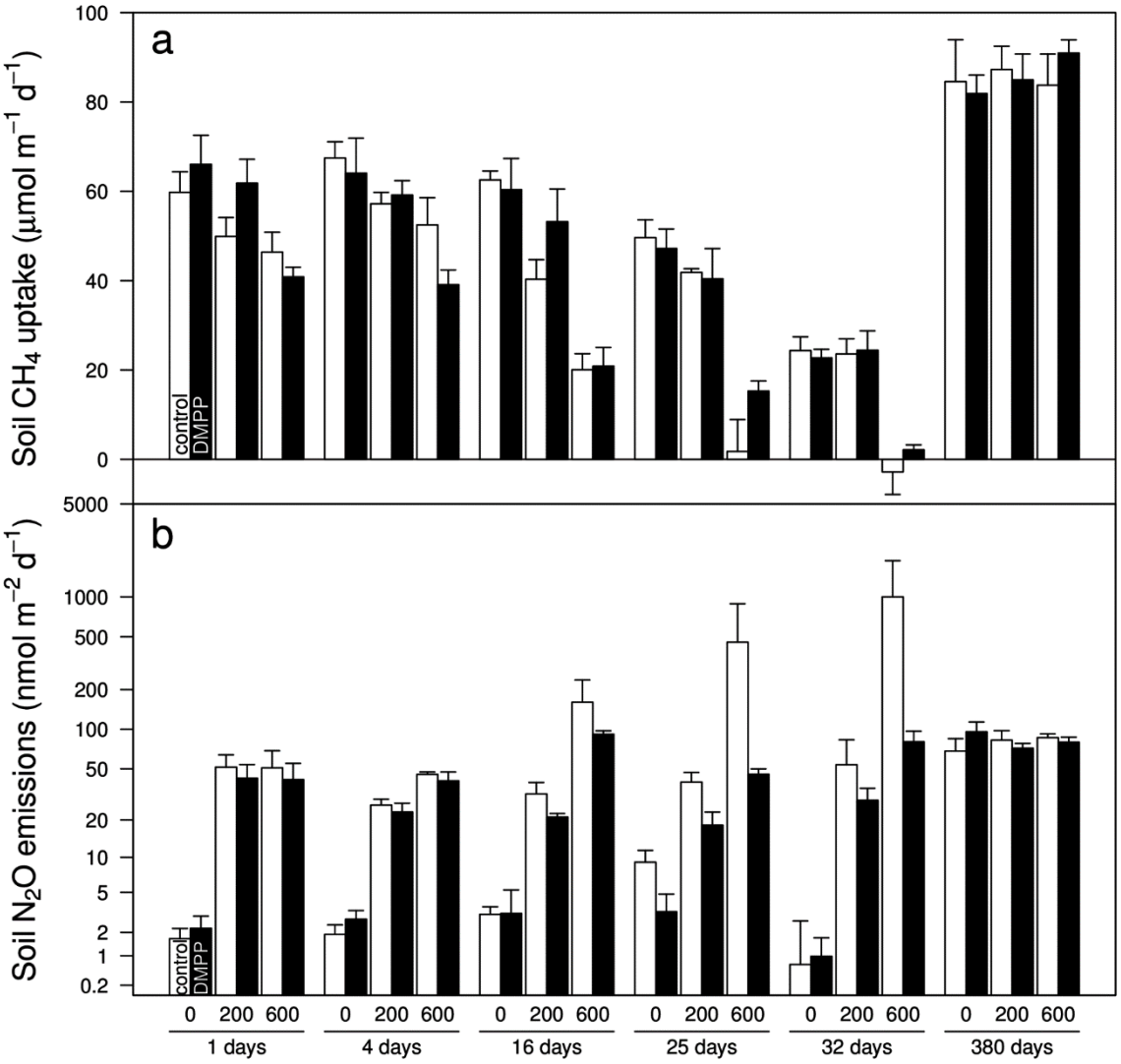
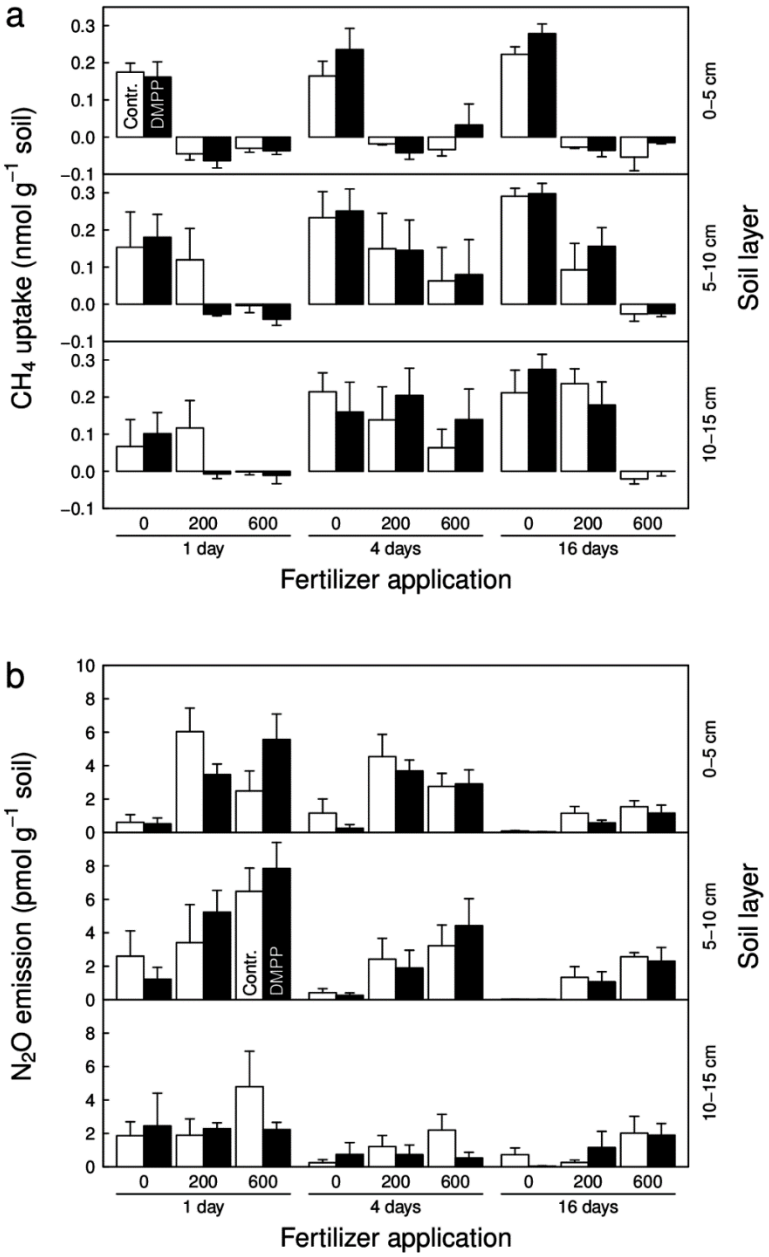


Fig. 1. Soil CH_4 uptake (a) and N_2O emission (b) measured using static chambers in the field experiment. Fluxes are shown in dependence of the application of N-fertilizer and nitrification inhibitor. Error bars indicate standard errors of means.



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462 Fig. 2. CH_4 uptake (a) and N_2O release (b) of sieved soil samples from different depths incubated
463 in the laboratory. Error bars indicate standard errors of means.

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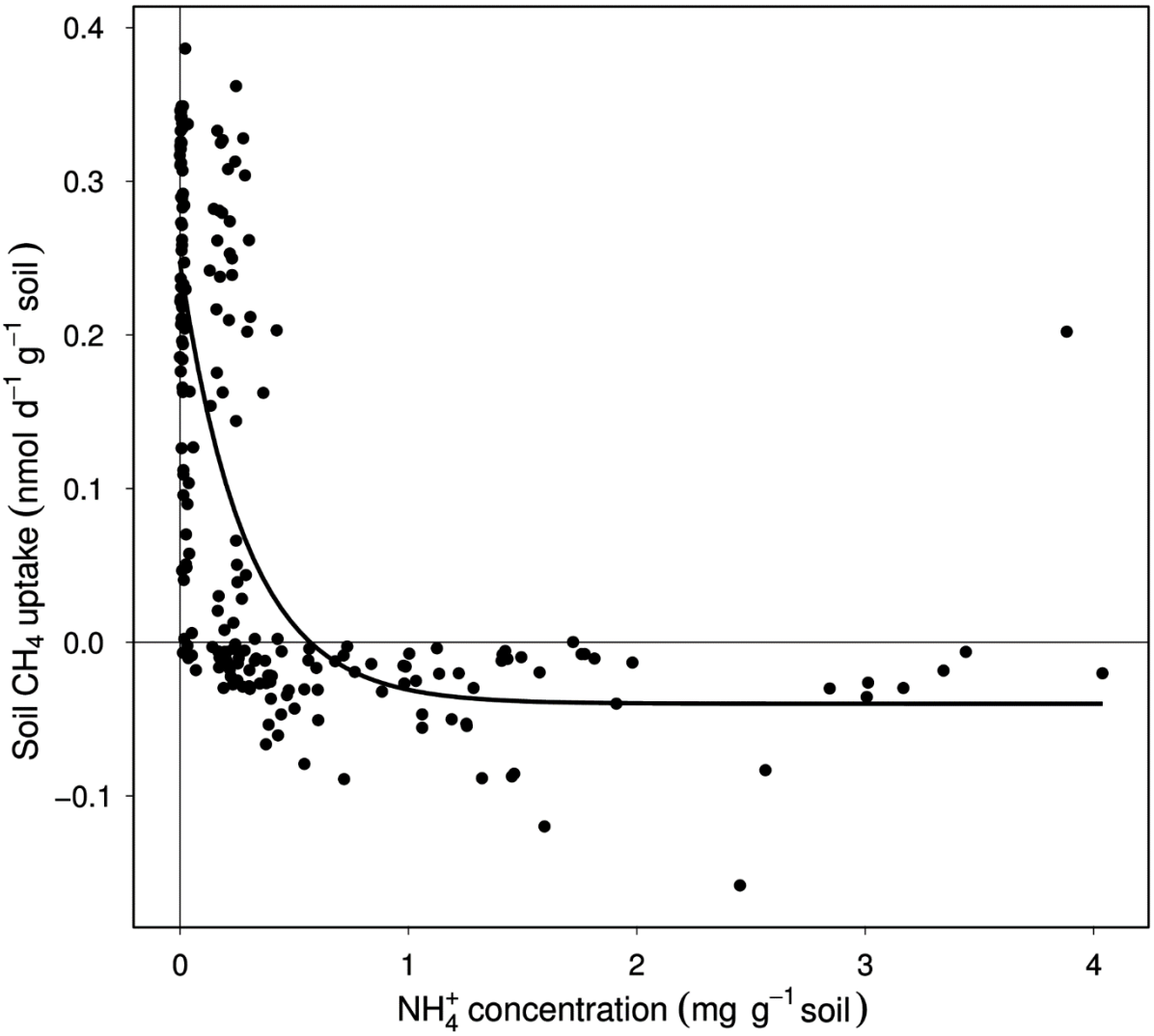
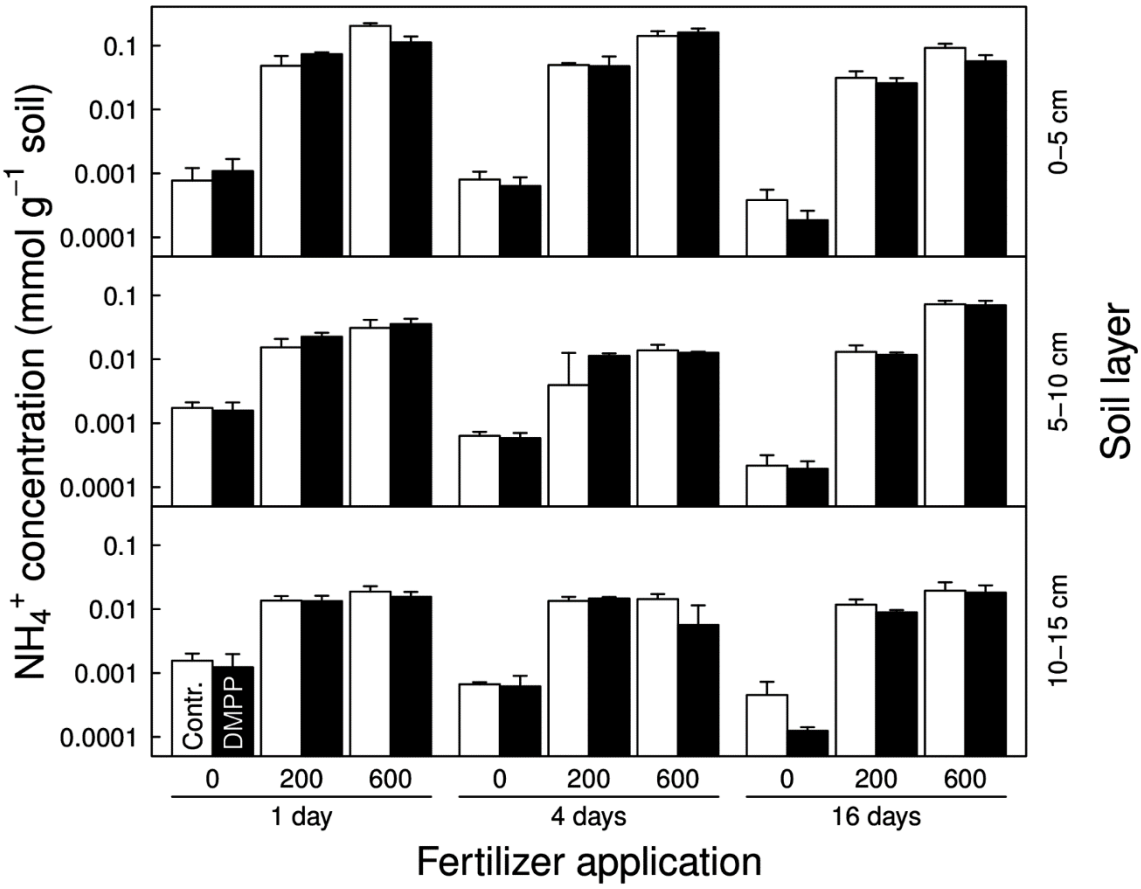


Fig. 3. CH_4 oxidation in dependence of NH_4^+ concentration measured in sieved soil samples. This figure combined all samples shown in Fig. 2, i.e. soil from all treatments, sampled 1, 4 and 16 days after treatment application, and from 0-5, 5-10, 10-15 cm soil depth.



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471 Fig. 4. NH_4^+ concentration in sieved soil samples as a function of treatments, sample collection
472 time, and soil depth. Error bars indicate standard errors of means.

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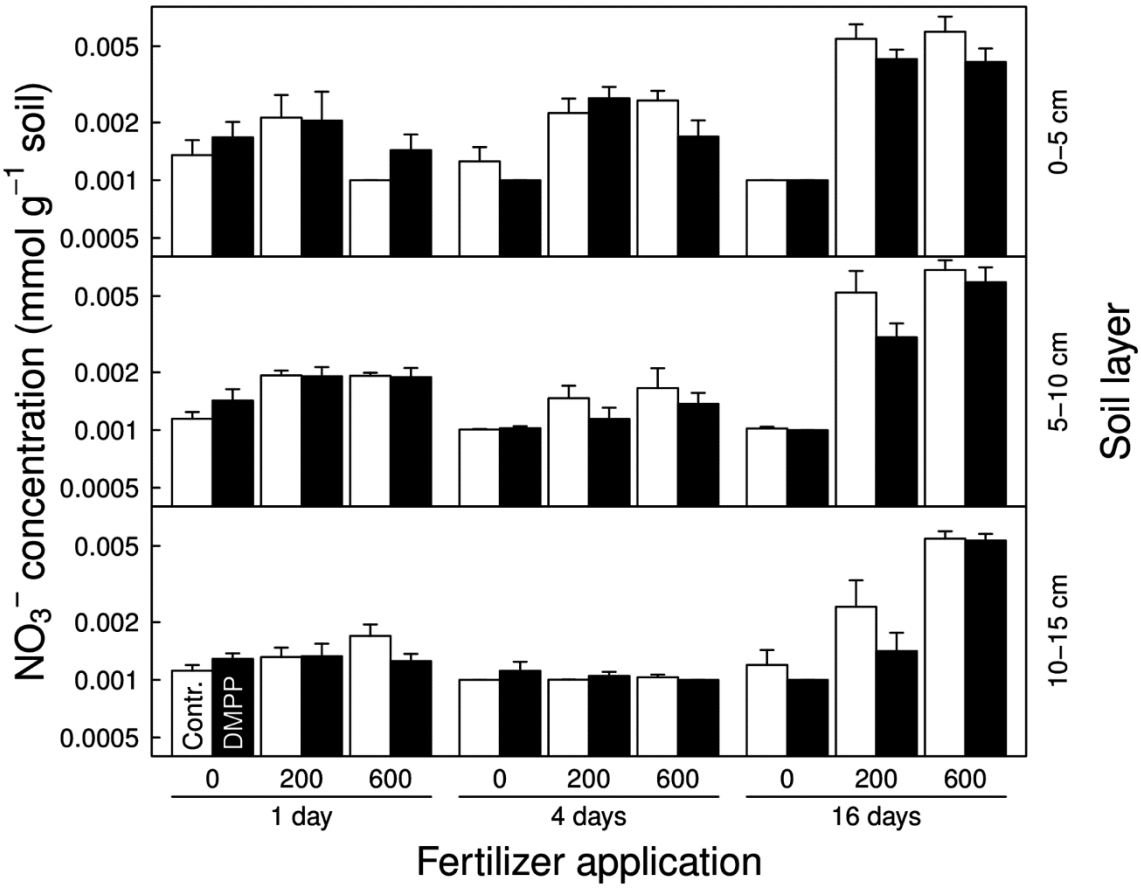

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475 Fig. 5. NO_3^- concentration in sieved soil samples as a function of treatments, sample collection
476 time, and soil depth. Error bars indicate standard errors of means.

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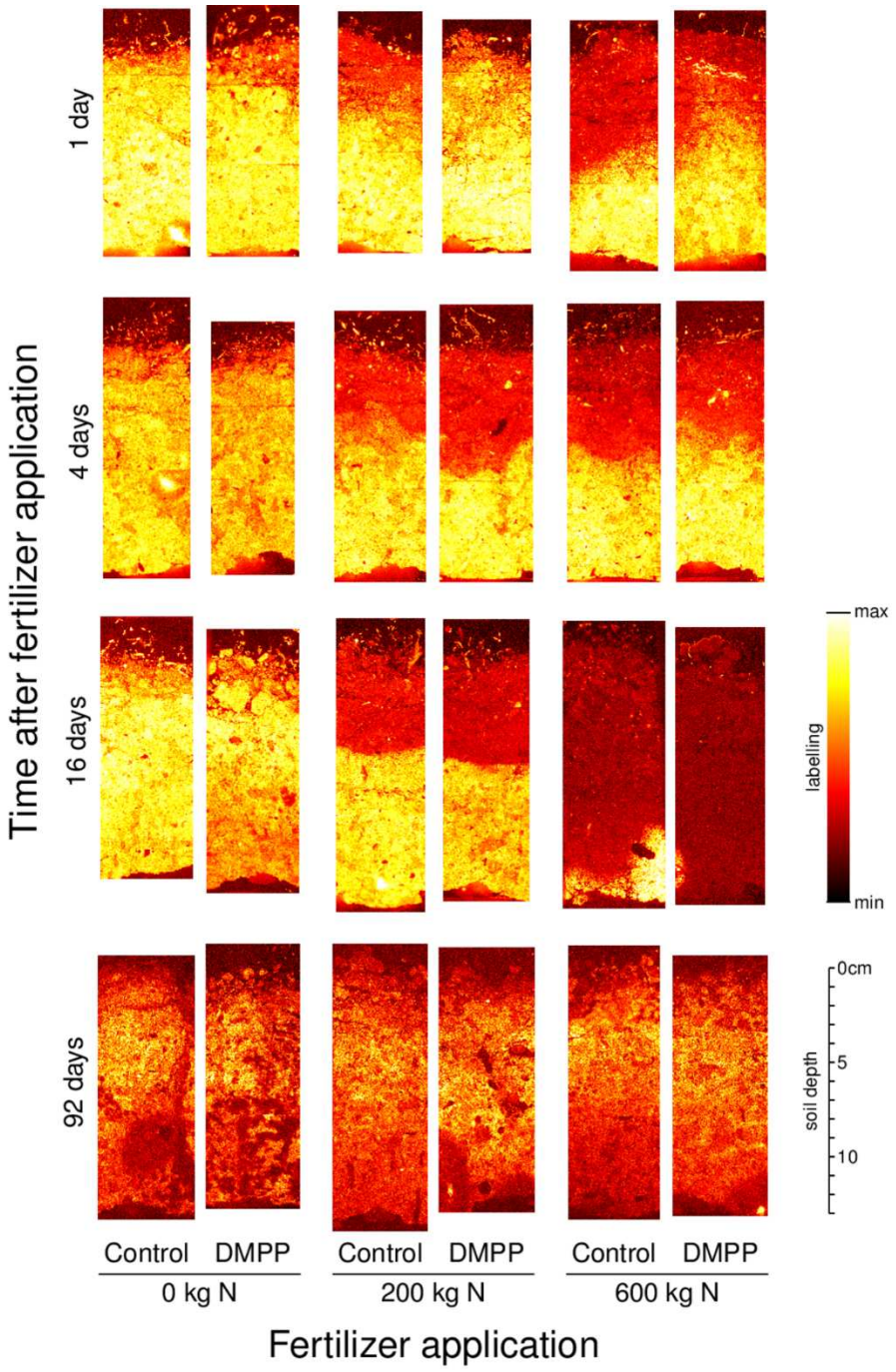

Fig. 6. Micro-autoradiographic images showing CH_4 assimilation by methanotrophs 1, 4, 16 and 92 days after the application of N fertilizer and nitrification inhibitor. Yellow indicates high ^{14}C activity whereas red to black indicates low ^{14}C labelling. Note that similar amounts of ^{14}C were applied to all soil samples; these figures thus reflect the relative label distribution within samples and do not allow to compare total methanotrophic activity of different plots. Data are shown for block 1.